AD NUMBER

AD835126

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Critical Technology; APR 1968. Other requests shall be referred to Department of the Army, Fort Detrick, Attn: Technical Releases Branch, Frederick, MD 21701.

AUTHORITY

Army Biological Defense Research Lab ltr dtd 29 Sep 1971

AD

TECHNICAL MANUSCRIPT 453

MECHANISM OF SYNTHESIS OF STAPHYLOCOCCAL ALPHA TOXIN

Charles W. Hendricks
Robert A. Altenbern

APRIL 1968

STATEMENT #2 UNCLASSIFIED

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701
attn: Technical Releases Branch

14

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 453

MECHANISM OF SYNTHESIS OF STAPHYLOCOCCAL ALPHA TOXIN

Charles W. Hendricks

Robert A. Altenbern

Medical Bacteriology Division
BIOLOGICAL SCIENCES LABORATORY

ABSTRACT

Several strains of <u>Staphylococcus aureus</u> were induced
with mitomycin C, a radiomimetic drug, and the results
rule out a general explanation for alpha toxin synthesis
based upon various aspects of lysogeny. Data are also
presented showing that toxin is generated intracellularly
and is continually released into the medium. Small
alkaline changes in pH by the metabolizing culture, not
affecting growth, are sufficient to stop or greatly
inhibit toxin synthesis. In an actively growing cul-
ture, this phenomenon appears as a sharp rise in
intracellular toxin synthesis, followed by a peak
and then decline, even though the culture continues
to grow and increase in optical density. Because
the toxin molecule is stable at normal cultural pH
values, an alkaline pH may interfere with toxin
synthesis at the intracellular level.

I. INTRODUCTION

Certain strains of <u>Staphylococcus</u> <u>aureus</u> are capable of producing alpha tox.n, which is lethal, dermatonecrotic, and hemolytic to erythrocytes of a number of animal species.[1,2] It is generally agreed that alpha toxin is a protein with a molecular weight of about 44,000 and is produced only when the culture is undergoing rapid cellular division.[3]

Specific mechanisms of alpha toxin induction, synthesis, and subsequent release from the cell are not clear, although Blair and Carr[4] postulated that lysogeny may play a role similar to that described by Barksdale, Garmise, and Rivera[5] for toxinogeny in <u>Corynebacterium</u> <u>diphtheriae</u>. The present investigation determined the extent to which lysogeny may be considered as a general explanation for alpha toxinogeny and clarified some of the factors involved in toxin synthesis and release.

II. MATERIALS AND METHODS

A. BACTERIAL STRAINS

The major portion of this investigation utilized the high alpha-toxin-producing <u>S</u>. <u>aureus</u> strain 233.* Eight alpha-toxin-negative mutants derived from strain 233 were obtained from the same source. Seventeen additional staphylococcal strains, which were originally recovered from clinical material at the Frederick Memorial Hospital Laboratory, were obtained from Fort Detrick sources. Stock cultures of all organisms were maintained on trypticase soy agar (BBL) slants at 4 C, and subcultures were made once every 3 months. Prior to an experiment, the organisms used were cloned on trypticase soy agar plates containing 5% rabbit blood in order to obtain a homogeneous population with respect to the toxin characteristics.

B. MEDIA

The liquid medium utilized in all experiments was essentially that of Coulter.[6] It consisted of 15 g proteose peptone (Difco B120) per liter, 5 g yeast extract (Difco) per liter, 2.5 ml lactic acid (Fisher), and distilled water to make 1 liter. The pH was adjusted to 7.6 with 1 M NaOH and the medium was autoclaved at 121 C for 20 minutes. Prior to use, a small amount of Dow-Corning Antifoam spray was added to each flask.

---

* Obtained from Dr. E.D. Rosenblum, University of Texas, Southwestern Medical School, Dallas, Texas.

C. ALPHA TOXIN ASSAY

Culture material for both intra- and extracellular alpha toxin assay was prepared by centrifuging (1,650 x g) 20 ml of the culture for 10 minutes. The resulting supernatant fluid was immediately decanted and titered for alpha toxin by the technique of Cooper, Madoff, and Weinstein[7] using twofold dilutions in phosphate buffered (pH 7.0) saline (0.155 M NaCl).

To obtain an estimation of intracellular alpha toxin activity, lyso-staphin* was added to the sedimented cells to a final concentration of 40 µg/ml, and the suspension was incubated for 1.5 hours at 37 C. After lysis, the final volume was adjusted to 1.5 ml with buffered saline, and the cellular debris was sedimented by centrifugation (1,650 x g) for 10 minutes. The resulting extract was titered for alpha toxin and assayed for protein by the procedure of Lowry et al.[8]

Stock solutions of mitomycin C** (0.1 mg/ml) were maintained in 0.1 M sodium phosphate buffer (pH 7.4) at 4 C.

III. RESULTS

A. MITOMYCIN-C-INDUCED LYSIS AND ALPHA TOXIN SYNTHESIS

Preliminary experiments showed that a final concentration of 1.0 µg mitomycin per ml produced optimal lysis in 2 to 4 hours at 37 C with sensitive strains. Of the 17 hospital isolates, 13 strains produced differing amounts of alpha toxin; however, only five of the 13 exhibited mitomycin-C-induced lysis. Strain 233 and all eight toxin-negative mutants derived from 233 were sensitive to mitomycin C, lysis normally occurring within 2 to 2.5 hours after induction.

Cells recovered by centrifugation from an overnight culture of S. aureus 233 were inoculated into 2 liters of Coulter's medium in prewarmed 4-liter Erlenmeyer flasks, to yield an initial optical density of 95 Klett units. The flasks were then incubated on a reciprocating shaker at 37 C in an air atmosphere. Samples were removed at 30-minute intervals and assayed for extracellular and intracellular alpha toxin, total protein, pH, and optical density. When the optical density reached 125 Klett units, the culture was divided into two equal portions; one received mitomycin C

---

* A gift from Dr. P.A. Tavormina, Mead Johnson Research Center, Evansville, Indiana.
** Obtained from the California Corporation for Biochemical Research.

(1.0 μg/ml), the other served as the control. Both cultures were returned
to the incubator-shaker and assayed periodically as described above.
Representative data are presented in Figure 1. Of considerable interest
is the observation that, in the control culture, intracellular toxin
appears suddenly and attains nearly maximal concentration within 1 hour.
The values plotted for intracellular toxin are specific activity values
(units per ml per mg protein) and show that the rate of synthesis of alpha
toxin far outstrips the rate of general protein synthesis. In this respect,
the phenomenon resembles an induction process. In contrast, extracellular
toxin appears somewhat later and increases relatively gradually throughout
the course of the experiment. It is clear that mitomycin C exerts a
pronounced inhibitory effect on growth and prevents appreciable formation
of either intracellular or extracellular alpha toxin.

B. MEDIUM pH AND ALPHA TOXIN SYNTHESIS

Experiments similar to the foregoing were conducted with cultures in
unbuffered Coulter's medium, and the pH of each sample was determined.
Typical data from such experiments (Fig. 2) demonstrate even more clearly
the rapid rise in intracellular toxin followed by the later appearance
and gradual increase in amount of extracellular alpha toxin. Of consider-
able pertinence is the fact that the total toxin in the culture (extra-
cellular plus intracellular) continued to rise after the peak intracellular
concentration had been obtained. In addition, the data strongly suggest
that toxin production in unbuffered Coulter's medium is markedly influenced
by pH of the culture. When the culture became more alkaline than pH 7.4,
toxin synthesis declined rapidly, even though growth proceeded at an
unaltered rate.

Consequently, experiments were performed in which growing cells of
strain 233 that had just initiated toxin production were resuspended in
Coulter's medium buffered (phosphate, 0.1 M) at specific pH values. The
results of these experiments (Fig. 3) show that a relatively acidic pH
(6.5) permits maximal toxin production. The medium adjusted to pH 7.9
supported only scant toxin production, and intracellular toxin synthesis
was almost completely shut off, even though the culture density increased
at the same rate as that in cultures grown in the medium buffered at the
lower pH values. These observations were confirmed when Coulter's broth
was initially buffered at various pH values and used as the growth medium.
Citrate-phosphate buffer was used for pH 4.3, sodium phosphate buffer for
pH values of 6.4, 6.9, 7.1, 7.2, 7.4, and 7.8, and Tris buffer for pH
values of 8.0 and 8.8. The final concentration of all buffers was 0.1 M.
No toxin was produced either intracellularly or extracellularly at pH
values of 4.3, 8.0, or 8.8, although optical densities were within 20 Klett
units of those cultures in which toxin was rapidly produced. A plot of
the maximal intracellular toxin concentration attained as a function of
the pH of the culture (buffered medium) shows the marked dependence of
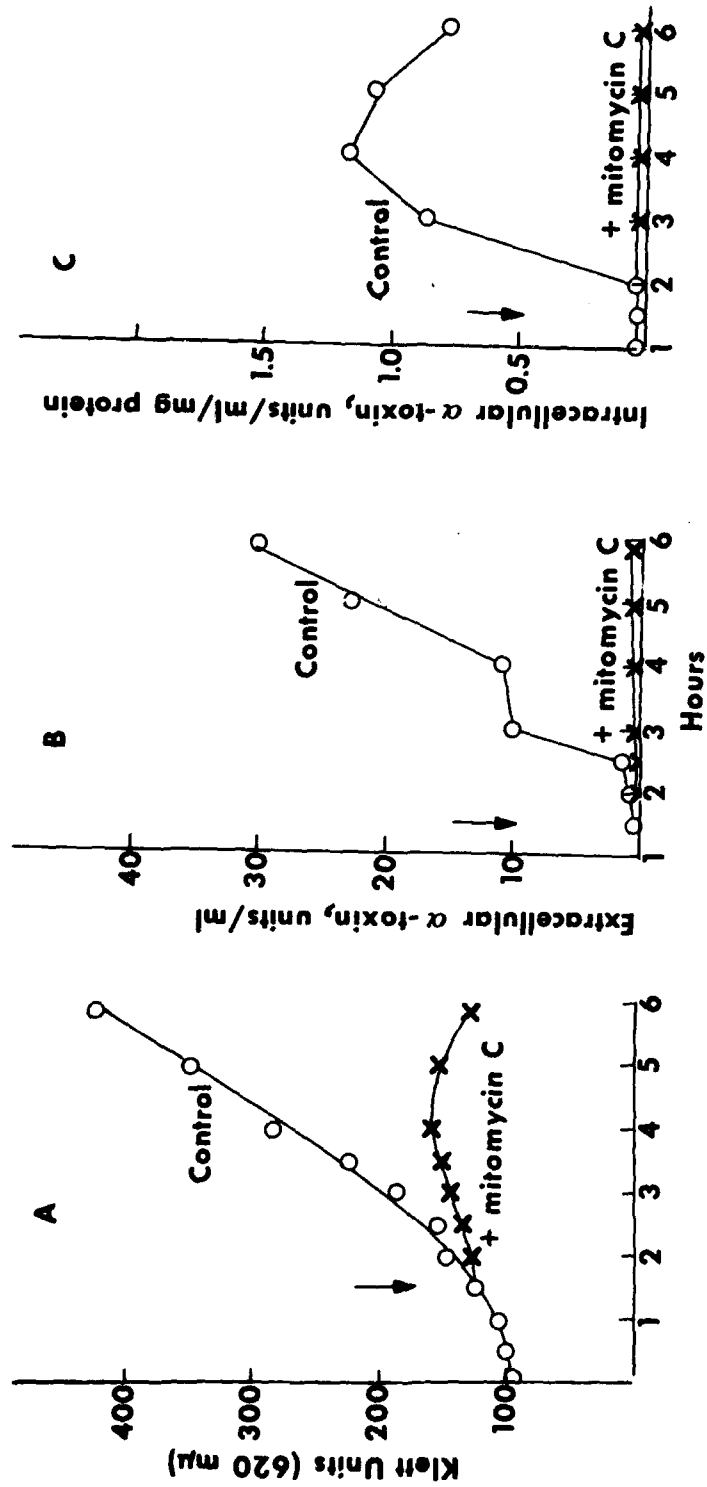toxin formation on medium pH (Fig. 4).

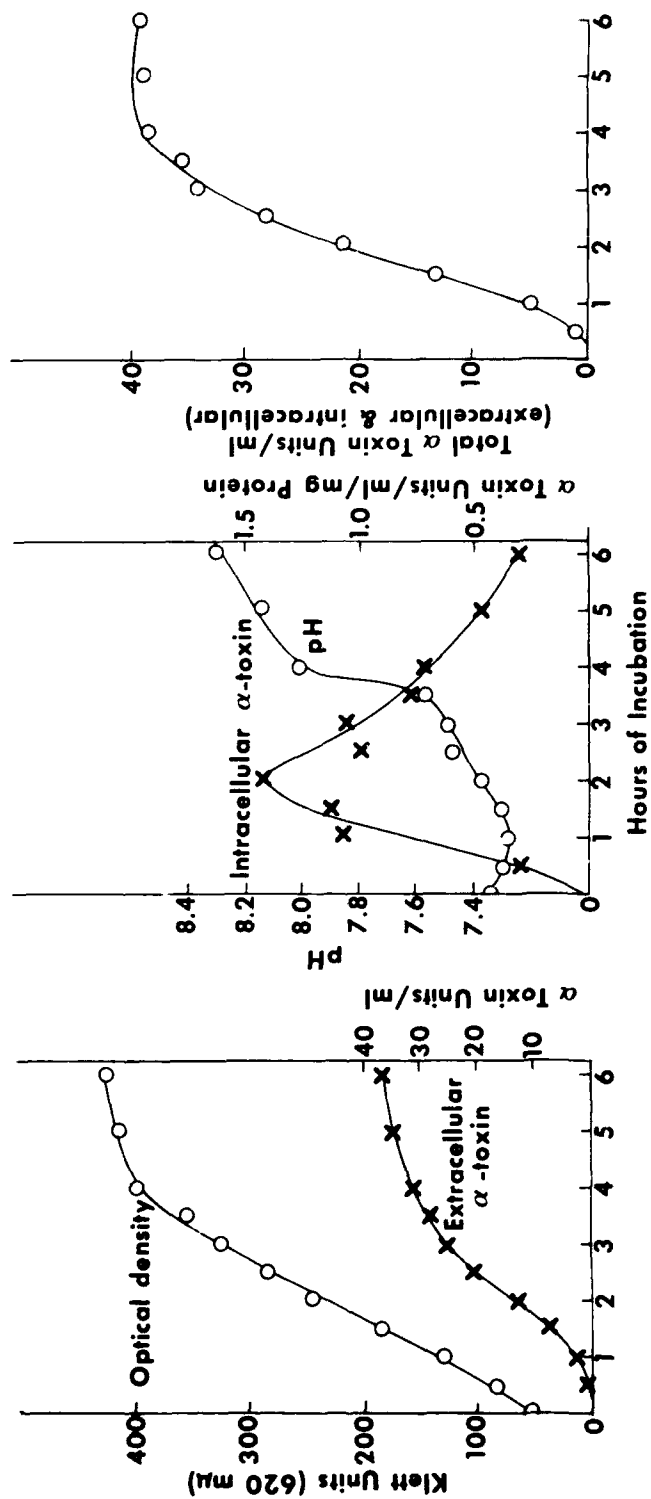Figure 1. Mitomycin C Induction of Lysis of S. aureus 233.

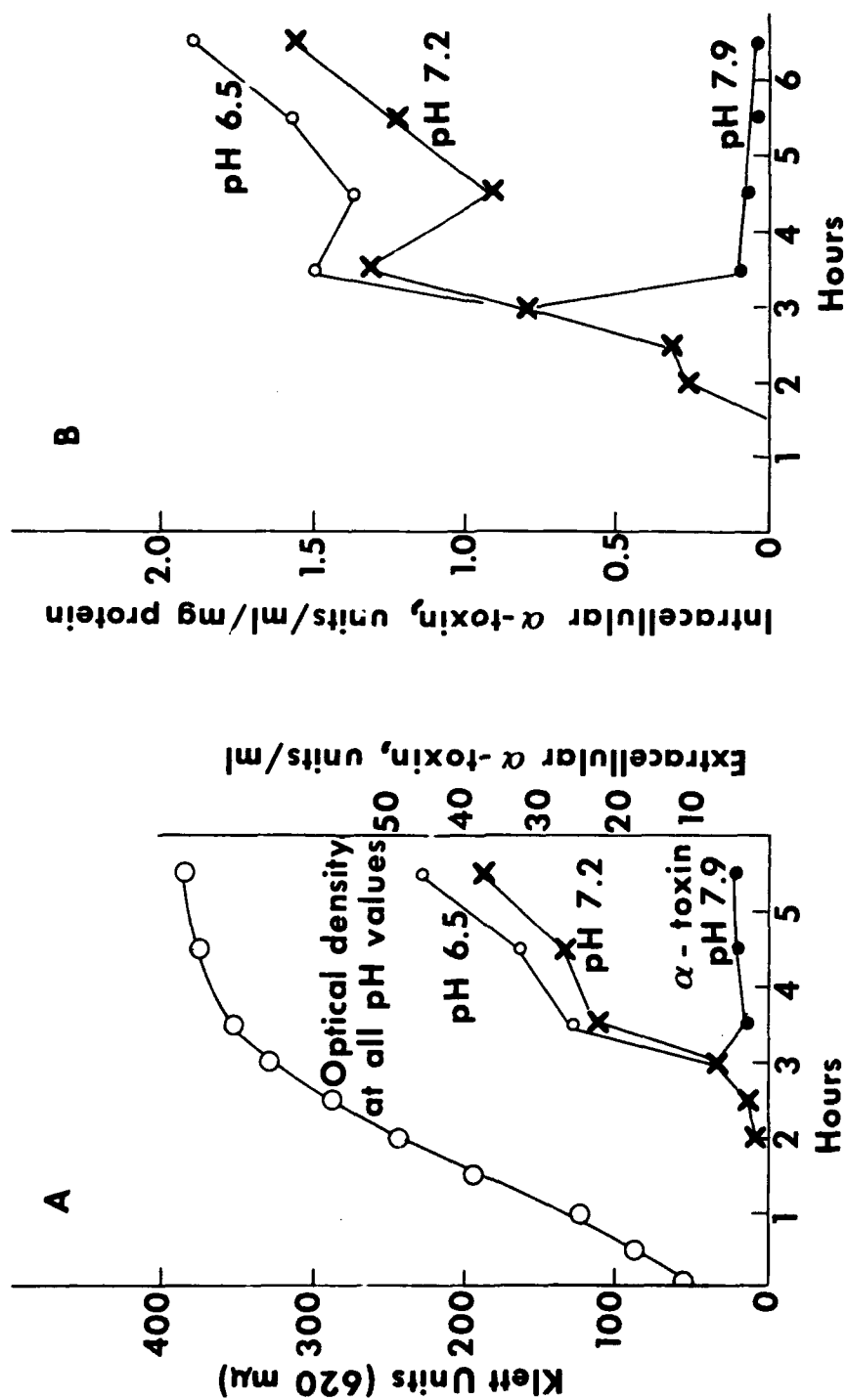Figure 2. Alpha Toxin Production in Unbuffered Coulter's Medium.

Figure 3. Alpha Toxin Production in Coulter's Medium Adjusted to Various pH Values.

C. IN VITRO COMPLEMENTATION

Attempts to demonstrate alpha toxin synthesis by in vitro complementation by both the adjacent-streak technique and mixing cell extracts were without major success. In a few experiments, an increase in titer, always by less than a factor of 2, occurred when extracts prepared from the parent strain 233 or one of the mutants were mixed with those from any other mutants. These titers did not increase with time as one might expect, and the small increases observed probably do not represent in vitro complementation.
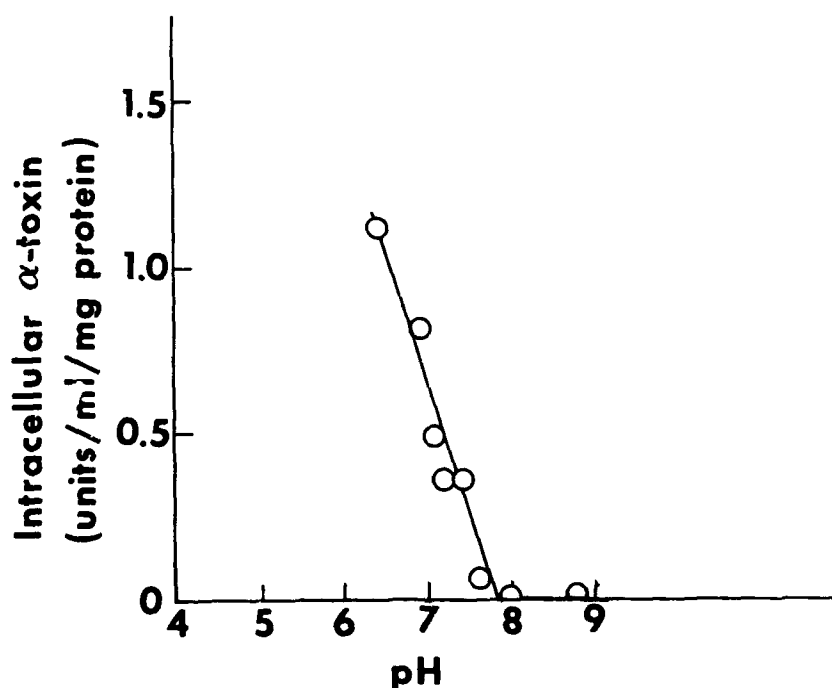

Figure 4. Alpha Toxin Production in Coulter's Medium
Buffered to Various pH Values.

IV. DISCUSSION

Specific mechanisms of alpha toxin synthesis and release have yet to be fully elucidated. From the data presented in this study, it is difficult to justify a general mechanism for alpha toxin synthesis in S. aureus based upon lysogeny. Not all of the alpha-toxin-producing strains were sensitive to mitomycin C, and the converse was also true. Because the induction of lysis by mitomycin C can offer presumptive evidence of lysogeny, even in the absence of a suitable indicator strain,[9] it appears doubtful that toxinogenicity results solely from lysogenic conversion. However, such a phenomenon has been described for the apparent linkage of the loss of beta toxin and gain of fibrinolysin characters in certain strains of S. aureus.[10] Data in the present study also eliminate from consideration a general synthesis mechanism similar to that in C. diphtheriae,[8] where diphtheria toxin is programmed by a bacteriophage genome. If prophage induction did play a decisive role in alpha toxin synthesis, a dramatically increased rate of toxin formation should have occurred on the intracellular level when the prophage was induced with mitomycin C. This did not occur, and the total toxin synthesized remained depressed.

The surprising behavior of intracellular toxin synthesis toward cultural pH in the uninduced control cultures is of considerable interest. A rapid rise in intracellular toxin was observed in these studies, followed by a peak and then a decline in synthesis, even though the culture continued to grow and increase in optical density. These results show clearly that, at some point in the growth history of the culture, alpha toxin synthesis is switched on or is greatly accelerated. The concept that alpha toxin is synthesized only during a short period followed by a switch off and external release to the medium can be discounted because the total toxin (intracellular plus extracellular) continues to rise long after the peak intracellular concentration is attained.

The sensitivity of production of staphylococcal products to abrupt or even gradual pH changes is not a new concept. A similar observation was made by Altenbern[11] for coagulase, which was not released from growing cells at slightly acidic pH values. Other experiments not presented here indicated that the active toxin molecule is stable at the pH range normally found in a growing culture. Thus, it is likely that toxin synthesis is affected at the intracellular level by pH change, although alkaline conditions might also inhibit the process of release of toxin into the medium.

Coulter[8] and McClatchy and Rosenblum[18] strongly suggested that two cistrons comprised the alpha toxin locus. In such a situation, one cistron could be transcribed and translated at an appreciable rate throughout growth, whereas the other cistron could be under very specific

pH control. However, in vitro complementation experiments, even with concentrated material, were unsuccessful under a wide variety of conditions, and this particular line of reasoning must be revised.

A great majority of enzymatically active proteins composed of subunits can be readily dissociated into the basic subunits by addition of 8 M urea. However, alpha toxin is completely unaffected by urea concentrations as high as 8 M or by sulfhydryl reductants such as dithiothreitol.* Thus, the alpha toxin molecule behaves as a single polypeptide chain, not dissociable into smaller units. Consequently, the two transductional groups reported by McClatchy and Rosenblum[12] may be a collection of mutants, each of which bears a mutation at one of two highly mutable points (hot-spots) in the alpha toxin gene.

LITERATURE CITED

1. Madoff, M.A.; Weinstein, L. 1962. Purification of staphylococcal alpha-hemolysin. J. Bacteriol. 83:914-918.

2. Bernheimer, A.W.; Schwartz, L.L. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. 30:455-468.

3. Elek, S.D. 1959. Staphylococcus pyogenes. E. and S. Livingstone, Ltd., London.

4. Blair, J.E.; Carr, M. 1961. Lysogeny in staphylococci. J. Bacteriol. 82:984-993.

5. Barksdale, L.; Garmise, L.; Rivera, R. 1961. Toxinogeny in Corynebacterium diphtheriae. J. Bacteriol. 81:527-540.

6. Coulter, J.R. 1966. Production, purification, and composition of staphylococcal α toxin. J. Bacteriol. 92:1655-1662.

7. Cooper, L.Z.; Madoff, M.A.; Weinstein, L. 1964. Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin: I. Kinetics of the lytic reaction. J. Bacteriol. 87:127-135.

8. Lowry, O.H.; Rosenbrough, N.J.; Farr, A.L.; Randall, A.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

9. Altenbern, R.A.; Stull, H.B. 1965. Inducible lytic systems in the genus Bacillus. J. Gen. Microbiol. 39:53-62.

10. Winkler, K.C.; de Waart, J.; Grootsen, C.J. 1965. Lysogenic conversion of staphylococci to loss of β-toxin. J. Gen. Microbiol. 39:321-333.

11. Altenbern, R.A. 1966. On the nature of albumin promoted coagulase release by Staphylococcus aureus. J. Infect. Dis. 116:593-600.

12. McClatchy, J.K.; Rosenblum, E.D. 1966. Genetic recombination between α-toxin mutants of Staphylococcus aureus. J. Bacteriol. 92:580-583.

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY *(Corporate author)*	2a. REPORT SECURITY CLASSIFICATION
Department of the Army Fort Detrick, Frederick, Maryland 21701	Unclassified
	2b. GROUP

3. REPORT TITLE

MECHANISM OF SYNTHESIS OF STAPHYLOCOCCAL ALPHA TOXIN

4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*

5. AUTHOR(S) *(First name, middle initial, last name)*

Charles W. Hendricks
Robert A. Altenbern

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
April 1968	15	12

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1B014501B71A	Technical Manuscript 453
c.	9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)*
d.	

10. DISTRIBUTION STATEMENT

Qualified requesters may obtain copies of this publication from DDC.
Foreign announcement and dissemination of this publication by DDC is not authorized.
Release or announcement to the public is not authorized.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Department of the Army Fort Detrick, Frederick, Maryland 21701

13. ABSTRACT

Several strains of *Staphylococcal aureus* were induced with mitomycin C, a radiomimetic drug, and the results rule out a general explanation for alpha toxin synthesis based upon various aspects of lysogeny. Data are also presented showing that toxin is generated intracellularly and is continually released into the medium. Small alkaline changes in pH by the metabolizing culture, not affecting growth, are sufficient to stop or greatly inhibit toxin synthesis. In an actively growing culture, this phenomenon appears as a sharp rise in intracellular toxin synthesis, followed by a peak and then decline, even though the culture continues to grow and increase in optical density. Because the toxin molecule is stable at normal cultural pH values, an alkaline pH may interfere with toxin synthesis at the intracellular level.

14. Key Words

Staphylococcus aureus
*Toxin
Hemolysins
Mitomycin C

DD FORM 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.